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(54) Title: NOVEL FACTOR IX PURIFICATION METHODS (57) Abstract Provided by the present invention are novel methods of factor IX protein recovery and purification, making use of anion exchange chromatography heparin-like resins, hydroxyapatite and immobilized metal affinity chromatography.		

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NOVEL FACTOR IX PURIFICATION METHODS

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FIELD OF INVENTION

The present invention relates generally to novel protein recovery and purification methods and more specifically to novel methods for the recovery and purification of factor IX.

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BACKGROUND OF THE INVENTION

The advent of recombinant technology now allows for the production of high levels of proteins within suitably transformed host cells. For secreted proteins, purification of the protein of interest involves isolation and purification from the host cell culture medium. Typically, the culture medium contains selected nutrients (*e.g.* vitamins, amino acids, cofactors, minerals,) and additional growth factors/supplements including insulin and possibly additional exogenous proteins. Conditioned medium contains not only the secreted product of interest, but also significant quantities of additional secreted host cell proteins and other substances (*e.g.* nucleic acids, membrane vesicles). Although expressed at high levels, the product of interest may represent a minority of all proteins present in conditioned medium. Not unexpectedly, proteins secreted by transformed host cells may possess characteristics quite different from those of the product of interest (*e.g.* charge, molecular size, amino acid composition). Similarly, selected secreted host cell proteins may exhibit properties very similar to those of the product of interest, thereby placing significant burden on the process used for purification. While developing a process for purification of a recombinant protein from conditioned medium, it is important that conditions used be limited with respect to denaturation of the product of interest (conditions which could be used to exploit minor differences between secreted proteins for major benefit to separation), thereby making it difficult to separate the product of interest from all other host cell proteins present.

In addition to secreted host cell proteins described above, conditioned medium may also contain products derived from the heterologously-expressed gene coding for the product of interest. These are not desirable for the final drug substance and include, for example, product forms lacking certain post-translational modifications such as glycosylation, sulfation, gamma carboxylation, or other modification potentially necessary for biological activity. In addition, proteolytically-degraded forms of the product of interest may be present in conditioned medium which also need to be removed during purification, but which very closely resemble the product of interest. Unfortunately, most approaches, such as ion exchange chromatography,

hydrophobic interaction chromatography, and size exclusion chromatography may not provide the extent of resolution of the product of interest necessary for use in human therapeutic situations from these undesired forms. To take full advantage of minor differences between the desired product and contaminants (*e.g.* small charge differences, small differences in molecular
5 size) the use of strong denaturants is often required. Such denaturants, however, can lead to loss of biological activity, expression of neoantigenic sites, and potentially enhance chemical decomposition of selected post-translational modifications.

In addition to separating the product of interest from molecules with similar properties (*e.g.* modified forms of the expressed gene), it is also important to recognize the need to
10 separate the desired product from components present in conditioned medium with which it specifically interacts. Where the protein of interest is positively charged, it will tend to bind to any negatively charged molecules present thereby making purification of the protein by traditional methods very difficult.

Of general background interest to the present invention are the following. Yan, USPN
15 4,981,952 (January 1, 1991) and Yan, *et al.* Bio/Technology 8:655 (July 1990) which disclosed the use of pseudo-affinity anion exchange chromatography for the purification of vitamin K-dependent proteins. Josic, *et al.* J. Chrom. 632:1 (1993) disclosed the use of heparin affinity chromatography to resolve factor IX from other vitamin K-dependent proteins. Suomela, Thromb. Res. 7:101 (1975); Suomela, Eur. J. Bio. Chem. 71:145 (1976); and Suomela,
20 Thrombos. Haemostasis. 35:211 (1976) described the use of hydroxyapatite in the separation of various clotting factors and factor IX plasma variants (based on charge differences due to variation in content of carbohydrate moieties, for example, sialic acid and galactose). However, Reekers, *et al.* Haemostasis 1:2 (1972) demonstrated the inability of hydroxyapatite to separate factors II, VII and IX from each other and from other plasma proteins. Schwinn,
25 *et al.* USPN 4,411,794 disclosed the partial purification of blood clotting factors using hydroxyapatite in the presence of calcium at a concentration of 50-200 mM. Feldman, *et al.* Biotech. Blood Proteins 227:63 (1993) and Roberts, *et al.* Vox Sang 67(suppl. 1): 69 (1994) disclosed the reduction of viral infectivity using acidification and copper-charged chelating Sepharose which resulted in low factor IX yields from human plasma.

30 Typically, researchers have used combinations of traditional chromatographic techniques to purify desired products. Often times, such techniques are not sufficient for purification of a product to the level of purity and consistency desired for a human therapeutic product. Researchers have attempted to overcome this difficulty by use of affinity chromatography wherein a protein of interest is bound to an immobilized ligand with which it

interacts specifically. Following appropriate washing, the desired product can be eluted by disruption of the ligand-protein interaction, often resulting in a significantly more pure eluate. However, in the instance of separation of a desired product from modified forms present in conditioned medium, single step affinity chromatographic techniques may not be sufficient, and
5 must be used in conjunction with other affinity resins and/or traditional separation techniques. Even high resolution affinity chromatography steps (*e.g.*, immunoaffinity purification using an immobilized monoclonal antibody) may not afford sufficient resolution of the desired product from other components due to common sites of interaction (*e.g.*, where an epitope which is present in the product of interest, is present as well in a proteolytically-degraded form of the
10 product).

Accordingly, there continues to exist a need in the art for protein purification methods that effectively overcome such difficulties.

BRIEF SUMMARY OF THE INVENTION

Provided by the present invention are methods for the purification of factor IX in a
15 solution comprising the steps of applying the solution containing factor IX to an anion exchange resin, washing said anion exchange resin with a solution having a conductivity that is less than required to elute factor IX from the resin, eluting said anion exchange resin with a first eluant to form a first eluate, applying said eluate to a heparin or heparin-like (*e.g.*, negatively charged matrix) resin, eluting said heparin or heparin-like resin with a second eluant to form a second
20 eluate, applying said second eluate to an hydroxyapatite resin, and then eluting said hydroxyapatite resin with a third eluant to form a third eluate containing the purified factor IX. Optionally, the first eluate can be applied to an hydroxyapatite resin. As yet another option, the method comprises the further steps of applying the third eluate to an immobilized metal affinity resin, and then eluting the immobilized metal affinity resin with a fourth eluant to form
25 a fourth eluate containing the purified factor IX. According to the methods of the invention, the factor IX can be either plasma-derived, expressed by cells in culture, or recombinantly produced as is known to one skilled in the art. Preferably, the first wash comprises a solution having a conductivity that is less than required to elute factor IX from the column and is generally greater than or equal to the conductivity of the load solution and of the first eluant
30 buffer; this conductivity is sufficient to remove a substantial proportion of those contaminating proteins that would otherwise be present in the first eluate. A suitable first wash comprises a salt solution such as sodium chloride, potassium chloride, sodium sulphate, sodium phosphate, or potassium phosphate, and optionally, may contain a suitable buffering agent. Suitable concentration ranges are those which are effective in removing contaminants without eluting

factor IX and include for example 25 mM to 200 mM salt, and preferably is 200 mM sodium chloride. The first eluant comprises a divalent cation such as calcium, magnesium, manganese, strontium, zinc, cobalt, and nickel; suitable concentration ranges are those which are effective in eluting factor IX, including for example a solution containing a buffering agent at pH about 5 8.0 such as Tris, in the range of 5 to 100 mM, preferably approximately 50 mM, a salt such as NaCl in the range of 50 to 250 mM, preferably 100 mM, and calcium chloride in the range of 5 to 20 mM, preferably approximately 10 mM.

Suitable anion exchange resins include those resins having a positively charged group such as diethyleaminoethane (DEAE), polyethyleneimine (PEI), and quaternary aminoethane (QAE) and include Q-Sepharose Fast Flow, DEAE-Sepharose Fast Flow, POROS-Q, Fractogel-TMAE, Fractogel-DMAE, and QAE-Toyopearl, with the preferred resin being Q-Sepharose Fast Flow (Pharmacia).

The second eluant can be a suitable salt in buffer, such as Tris with sodium chloride and potassium chloride, with 50mM TRIS, 0.50M NaCl, pH 8.0 being preferred. Suitable heparin or heparin-like resins include those resins having a negatively charged group such as heparin, sulfated esters of cellulose, sulfolpropyl (SP), carboxyl, and carboxy methyl and include Matrex Cellufine Sulfate, Heparin Sepharose, Heparin Toyopearl, Carboxy Sulfon, Fractogel EMD-SO₃, and Fractogel-EMD COO, with the preferred being Matrex Cellufine Sulfate.

The third eluant can be a salt, such as phosphate and sulphate, with 0.5M potassium phosphate, 0.2M NaCl, pH 7.2 preferred. Suitable hydroxyapatite resins include any containing calcium-phosphate such as ceramic-Hydroxyapatite, Biogel HT, and others, with ceramic-HA preferred. The immobilized metal affinity resin can be one such as Fractogel-EMD-Chelate, Chelating-Sepharose, Matrex Cellufine Chelate, and POROS 20MC, with Fractogel EMD-Chelate currently preferred. The fourth eluant is a buffer solution containing a chelator such as imidazole, EDTA, EGTA, glycine, histidine, and Tris, with the preferred being 20 mM potassium phosphate, 15 mM imidazole, 0.1 M NaCl, pH 7.1.

Also provided by the present invention are factor IX compositions produced by the methods of the invention. The factor IX so produced has a specific activity in the range of 240-400 U/mg, and is optionally about 240 U/mg.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "factor IX" includes, but is not limited to factor IX isolated from plasma, transformed cell lines, and recombinantly produced factor IX isolated from host cell culture medium.

As used herein, the term "anion exchange resin" includes, but is not limited to resins having a positively charged moiety (at neutral pH), such as diethyleaminoethane (DEAE), polyethyleneimine (PEI), and quaternary aminoethane (QAE) and includes, for example, Q-Sepharose Fast Flow (Pharmacia), DEAE-Sepharose Fast Flow, DEAE-Toyopearl, QAE-Toyopearl, POROS-Q, Fractogel-DMAE, Fractogel EMD-TMAE, Matrex Cellufine DEAE and the like.

As used herein, the term "first wash" includes, but is not limited to a solution having a conductivity that is less than required to elute factor IX from the anion exchange column and whose conductivity is generally greater than or equal to the conductivity of the load solution and of the conductivity of the first eluant; this conductivity is sufficient to remove a substantial proportion of those contaminating proteins that would otherwise be present in the eluate. As one skilled in the art readily appreciates, the first wash can be any salt solution and includes, for example, sodium chloride, potassium chloride, sodium sulphate, sodium phosphate, or potassium phosphate, and can be suitably buffered. Typically, concentrations range from low (25 mM salt) to high (200 mM salt), with 200 mM sodium chloride presently preferred.

As used herein, the term "first eluant" includes, but is not limited to: solutions composed of a buffering agent (*e.g.* Tris) at a concentration of approximately 0.05M, salt (*e.g.* NaCl) at a concentration which is not sufficient for elution from the resin in the absence of divalent cation (*e.g.* approximately 0.10M-0.20M), and divalent cation (*e.g.* CaCl_2) at low concentrations of approximately 0.01M, at pH 8.0. The selection of buffer composition is compatible with the presence of divalent cation. Preferably, the "first eluant" has a lower conductivity than the "first wash".

As used herein, the terms "heparin" resin and "heparin-like" resin are used interchangeably, and include but are not limited to, resins containing an immobilized negatively charged moiety such as heparin, sulfated esters of cellulose, sulfolpropyl (SP), carboxyl, and carboxy methyl and includes Fractogel-EMD-SO₃, Carboxy Sulfon, Fractogel-EMD-COO, Heparin-Sepharose, and Matrex Cellufine Sulfate.

As used herein, the term "second eluant" includes, but is not limited to: solutions composed of a buffering agent (*e.g.* Tris) at a concentration of approximately 0.05M, and salt (*e.g.* NaCl, KCl, Na₂SO₄) at a concentration sufficient to disrupt the interaction of factor IX with the negatively-charged resin support (*e.g.* 0.50 M) at approximately pH 8.0. As used in this process, the second eluant should be compatible with the subsequent process step, *i.e.*, hydroxyapatite.

As used herein, the term "hydroxyapatite column" includes, but is not limited to: calcium phosphate gel supports including for example, BioGel-HT, and Ceramic-hydroxyapatite.

As used herein, the term "third eluant" (and "second phosphate buffer") includes, but is not limited to: solutions composed of a buffering agent (*e.g.* phosphate or sulfate) at concentrations sufficient to disrupt interaction of factor IX with the resin (*e.g.* approximately 0.20 M or higher) and salt (*e.g.* NaCl, KCl) present at concentrations sufficient to minimize charge-interactions of the factor IX with the hydroxyapatite resin, at approximately neutral pH (pH 7.2); the term "first phosphate buffer" includes but is not limited to solutions composed of a buffering agent (*e.g.*, phosphate or sulfate) at concentrations sufficient to remove inactive forms of factor IX from the hydroxy-apatite resin.

As used herein, the term "immobilized metal affinity resin" (IMAC) includes, but is not limited to: resins containing an immobilized functional moiety (*e.g.* iminodiacetic acid) capable of binding and coordinating multivalent cations including Chelating-Sepharose, Fractogel-EMD-Chelate, POROS 20MC, and Matrex Cellufine Chelate. The bound metal ion can be selected from several possible choices including but not limited to copper, nickel, cadmium, cobalt, iron, zinc, or strontium.

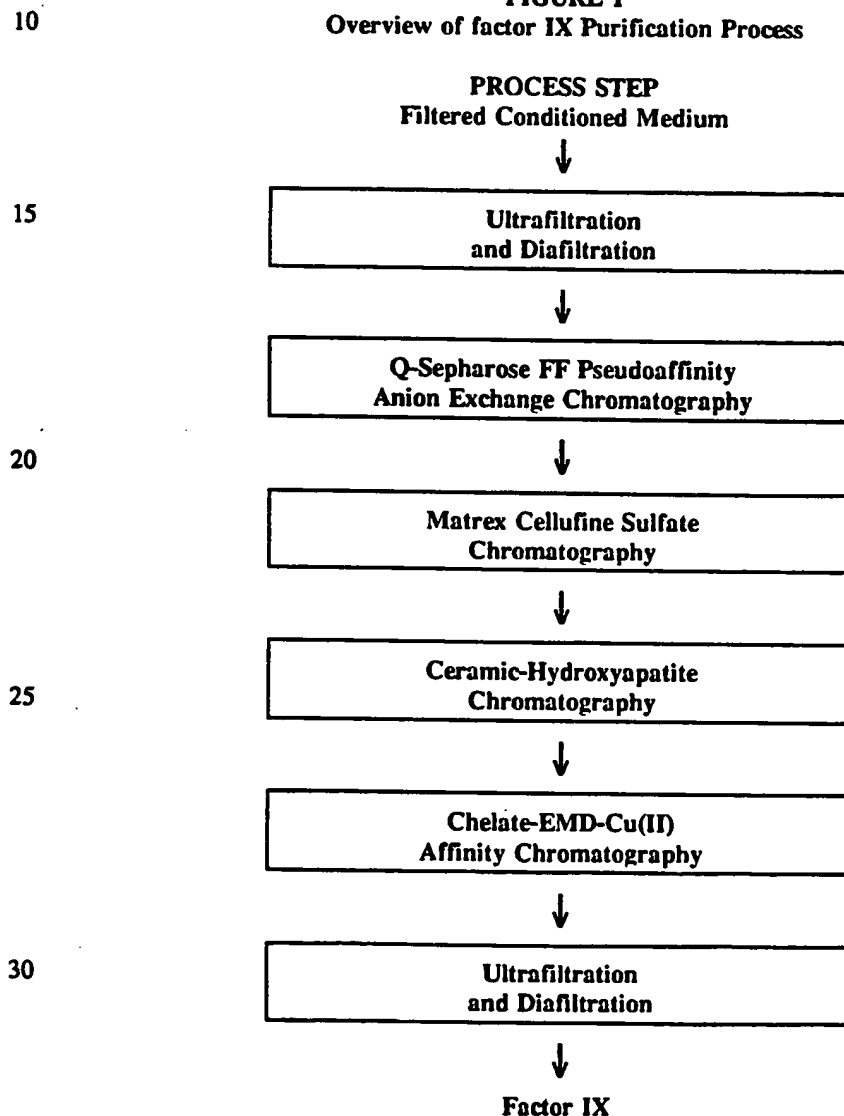
The term "fourth eluant" (also termed "displacer") includes but is not limited to, any compound which will displace bound factor IX from the IMAC resin support, while minimizing displacement of the immobilized metal ion from the resin support, and includes but is not limited to such compounds as glycine, histidine, tris, imidazole, EDTA, EGTA, and the like. As one skilled in the art readily appreciates, the appropriate concentration of displacer will vary according to binding affinity and can be ascertained by experimental evaluation of conditions. Typically, concentrations range from low (*e.g.* 5-15 mM displacer) to high (*e.g.* 100-200mM displacer).

Reference to factor IX specific activity of "U/mg" includes but is not limited to: biological activity determined in the *in vitro* (APTT) clotting assay using pooled plasma or isolated, purified factor IX as standard. The concentration of protein can be determined by any of several appropriately validated methods including SEC, RP-HPLC, dye-based assays (*e.g.*, Bradford, Lowry) or absorbance at 280 nm. Factor IX activity is determined according to the method of Pittman, D., *et al.*, Blood 79:389-397 (1992) utilizing factor IX-deficient plasma.

Figure 1 provides an overview of the process. While the order of the steps set forth is the presently preferred embodiment, it will be appreciated by one skilled in the art that the order can be re-configured if desired and that steps can be omitted.

According to the present invention, cells are first removed from conditioned medium, *e.g.* by microfiltration (MF) utilizing tangential flow filtration membranes with pore size of approximately 0.6 μ m. Optionally, cell-free conditioned medium is prepared for purification by filtering through a 0.45 μ m depth filter. The cell-free conditioned medium can then be concentrated by ultrafiltration, if desired, followed by diafiltration into an appropriate buffer for loading onto the first chromatographic step. Alternatively, the cell-free conditioned medium may be loaded directly onto the first chromatography column equilibrated in an appropriate buffer.

FIGURE 1
Overview of factor IX Purification Process



The initial process step, UF/DF#1, entails concentration of the cell-free factor IX-containing conditioned media by ultrafiltration, followed by diafiltration. Although not required for binding of factor IX to the first chromatography column, this step is effective in removing small-molecular-weight cell culture media components. Such components may bind to the initial chromatography column, thereby decreasing the capacity of the column for factor IX. UF/DF#1 is used to exchange the factor IX into an appropriate buffer solution for subsequent processing.

In the first chromatography step, anion-exchange on Q-Sepharose Fast Flow (FF) (Pharmacia), the factor IX is captured and purified from host-cell components present in the UF/DF #1 concentrated pool. The Q-Sepharose FF column adsorbs the factor IX protein, and contaminating host-cell proteins with isoelectric points greater than the operating pH are removed from the process stream by flowing through the column. The column to which factor IX is adsorbed is then washed prior to elution to remove loosely-bound contaminants and adjust the conductivity of the buffer in preparation for elution.

Typically, bound proteins are eluted from Q-Sepharose FF by increasing the ionic strength of the buffer. The factor IX purification process, however, employs this resin in a pseudo-affinity anion-exchange mode in which active factor IX is eluted by addition of *e.g.*, calcium chloride to the buffer. This divalent cation results in elution of active forms of factor IX from the resin. Some less active forms of factor IX may also elute from the Q-Sepharose FF column with this elution buffer. Selected inactive forms of factor IX and other, contaminating host-cell proteins remain bound to the column. The Q-Sepharose FF step achieves a significant increase in the purity of the factor IX.

In the second chromatography step, the Q-Sepharose FF elution pool is loaded directly, without dilution, onto the Matrex Cellufine Sulfate column. The factor IX adsorbs to the column, while other, contaminating proteins (*e.g.*, soluble PACE and other host-cell proteins present in the Q-Sepharose FF eluate) are removed from the process stream by flowing through the column. The column is washed with a low-ionic-strength buffer to remove all non-binding proteins. The factor IX is eluted by an increase in the ionic strength of the buffer, using salt (*e.g.*, sodium chloride).

Further removal of inactive factor IX forms is obtained during the third chromatography step, Ceramic-HA column chromatography. The pH of the Matrex Cellufine Sulfate elution pool is adjusted to approximately 7.5, and the elution pool is then loaded directly onto the Ceramic-HA column. The factor IX is adsorbed by the column. The Ceramic-HA column is washed with buffer to remove loosely bound contaminants, followed

by a wash with 50 mM potassium phosphate, 0.185 M NaCl (pH 7.2) to remove more tightly bound contaminants, including inactive forms of factor IX. Bound, active factor IX is eluted in a step-wise manner using a solution containing a higher concentration of potassium phosphate (*e.g.* 200mM or greater, pH 7.2) as the eluant.

5 The fourth chromatography step, Fractogel EMD-Chelate -Cu(II) chromatography, removes low levels of contaminating host-cell proteins still present in the product stream. The Ceramic-HA elution pool is loaded directly onto the Fractogel EMD-Chelate -Cu(II) column. Factor IX and a number of contaminating proteins are adsorbed to the column. Purified active factor IX is eluted from the column by low concentrations of imidazole (*e.g.* approximately
10 15mM) in the buffer, and the residual, contaminating host-cell proteins are removed from the product stream by remaining bound to the column.

Finally, the Fractogel EMD-Chelate -Cu(II) elution pool is concentrated by ultrafiltration, followed by diafiltration (UF/DF#2) into a buffer identical to a formulation buffer except that it does not contain polysorbate 80. A suitable formulation buffer comprises
15 histidine, glycine, sucrose, and polysorbate-80 optionally at 10mM, 260mM, 1 %, and 0.005 %, respectively. Upon completion of the diafiltration, factor IX is concentrated to achieve a target concentration. The product pool is removed from the UF/DF 2, apparatus and formulated by addition of polysorbate 80 to a target concentration of 0.005 %. The factor IX drug substance is then filtered (0.2 μ m), sampled, labeled, and stored frozen at approximately -80 °C. The
20 last process step, UF/DF#2, is effective in concentrating and diafiltering the purified factor IX drug substance without significant protein denaturation or loss. SDS-PAGE analysis (reduced and nonreduced) is one method used to evaluate overall process performance. Each step provides greater than 80% to 100% yield and the average overall yield of factor IX is about 51%. The overall process yield is determined from the clotting activity entering the
25 purification process and the total clotting activity in the factor IX drug substance (excluding material removed as in-process samples and retains).

The following examples illustrate practice of the invention. These examples are for illustrative purposes only and are not intended in any way to limit the scope of the invention claimed.

30 Example 1 describes concentration of protein by ultrafiltration/diafiltration; Example 2 relates to purification of factor IX by pseudo-affinity anion-exchange chromatography on Q-sepharose fast flow; Example 3 describes purification of factor IX by chromatography on Matrex Cellufine Sulfate; Example 4 relates to purification of protein with hydroxyapatite chromatography; Example 5 describes purification of protein by immobilized metal affinity

chromatography; and Example 6 relates to concentration and formulation of protein by ultrafiltration/diafiltration.

Example 1 - Concentration of Protein by Ultrafiltration/Diafiltration

Optionally, ultrafiltration/diafiltration (UF/DF#1) can be performed to concentrate and to buffer-exchange the cell-free conditioned medium using tangential-flow membrane filtration. The membrane used in the tangential-flow device serves as a selectively permeable filter that separates substances on the basis of molecular weight. Solution components of high molecular weight, such as factor IX, are retained by the membrane, and components of low molecular weight, such as inorganic salts and buffer components, pass freely through the porous membrane structure and are removed in the permeate.

When buffer is drawn from the tangential-flow device at a rate faster than that at which replacement buffer is added to the retentate, the protein solution is concentrated. When replacement buffer is added to the tangential-flow retentate at a rate approximately equal to the rate at which the buffer is drawn through the membrane, the initial buffer is continuously diluted (protein diafiltration). Under these conditions, compounds of low molecular weight are readily exchanged and the protein concentration remains constant. The addition of five retentate volumes of buffer results in a theoretical replacement of $\geq 99\%$ of the initial buffer.

Before use, the UF/DF#1 system is equilibrated with 50 mM TRIS, 150 mM NaCl, pH 7.5. The cell-free conditioned medium is concentrated approximately 20-fold relative to the initial volume of the cell-free conditioned medium. The concentrated cell-free conditioned medium is then diafiltered into the buffer. The diafiltration is complete when at least five retentate volumes of the buffer have passed through the membrane, resulting in a theoretical removal of $\geq 99\%$ of salts and other low-molecular-weight components present in the cell-free conditioned medium.

Once diafiltration has been completed, the retentate is concentrated if necessary. The equipment is then flushed with sufficient buffer to recover residual factor IX product from the reservoir and tubing. The pool is then pumped out of the UF/DF vessel and filtered through an autoclaved 0.2- μ m filter into a clean vessel. The UF/DF#1 pool is stored at 2 to 8 °C until it is further processed.

Example 2 - Purification of factor IX by Pseudo-Affinity Anion-Exchange Chromatography on Q-Sepharose Fast Flow

Q-Sepharose Fast Flow (FF) (Pharmacia) is a strong anion-exchange resin composed of a cross-linked agarose matrix that is covalently derivatized with a quaternary amine group through a short linker. Acidic proteins (such as factor IX) and other polyanionic substances with

a net negative charge at the pH of operation bind to Q-Sepharose FF via charge interactions. Typically, bound components are differentially eluted from Q-Sepharose FF by disruption of these charge interactions with solutions of increased conductivity. However, the factor IX purification process employs the Q-Sepharose FF resin in a pseudo-affinity mode. Factor IX is eluted from the column using a solution containing low-concentration (10 mM) calcium chloride. The inclusion of calcium ions in the elution buffer causes a conformational change in the factor IX that results in elution from the resin.

Q-Sepharose FF is used to capture factor IX from UF/DF#1 retentate; to remove uncharged and basic contaminants from the process stream (in the unbound fraction during loading of the column); to separate factor IX from acidic proteins (which bind to the resin but are not eluted by addition of calcium chloride to the buffer), including inactive forms of factor IX; and to deliver a concentrated factor IX process stream into the subsequent purification process chromatography step, Matrex Cellufine Sulfate.

Optionally, all chromatography operations for this step are performed at 2° to 8° C. The Q-Sepharose FF column is first charged with 50 mM TRIS, 2 M NaCl, pH 8.0, followed by equilibration with 50 mM TRIS, 150 mM NaCl, pH 8.0. The UF/DF#1 retentate is loaded onto the Q-Sepharose FF column, and the column is then washed with 50 mM TRIS, 200 mM NaCl, pH 8.0. This first wash ensures that the entire load has passed through the column and that non-adsorbing impurities in the load, as well as contaminants that are loosely bound to the resin, have been washed from the system. The column is then washed with 50 mM TRIS, 100 mM NaCl, pH 8.0 to lower the conductivity in preparation for elution.

Factor IX is eluted from the column with 50 mM TRIS, 100 mM NaCl, 10 mM CaCl₂, pH 8.0, and the eluted product is collected as a single peak. The Q-Sepharose FF eluate is sampled and stored at 2 to 8 °C until it undergoes further processing. Optionally, the column can be regenerated and reused.

Example 3 - Purification of factor IX by Chromatography on Matrex Cellufine Sulfate

Matrex Cellufine Sulfate is composed of spheroidal cellulose beads derivatized with sulfate esters. It can be used as an immobilized heparin analogue for affinity-purification of proteins containing heparin-binding domains. It can also be used for cation-exchange chromatography because of its negatively charged sulfate functions. Basic proteins, other polyionic substances with a net positive charge at the pH of operation, and heparin-binding

proteins bind to the resin and are eluted with solutions of increasing ionic strength. The Matrex Cellufine Sulfate resin is used in the factor IX purification process for removal of host-cell proteins other than factor IX in the Q-Sepharose FF elution pool and, optionally, to provide appropriate buffer conditions for loading the hydroxyapatite column.

5 Optionally, all chromatography operations for this step are carried out at 2 to 8 °C. In preparation for the load step, the Matrex Cellufine Sulfate column is equilibrated with 50 mM TRIS, pH 8.0. The Q-Sepharose FF elution pool is loaded directly onto the equilibrated Matrex Cellufine Sulfate column, the column is washed with 50 mM TRIS, 150 mM NaCl, 10 mM CaCl₂, pH 8.0 to ensure that all of the load has passed through the column and that weakly
10 bound impurities are removed from the system. Next, the column can be washed to remove calcium ions prior to elution.

After the wash steps have been completed, the Matrex Cellufine Sulfate column is eluted with 50 mM TRIS, 500 mM NaCl, pH 8.0, and the eluate collected as a single UV-absorbing elution pool. The Matrex Cellufine Sulfate elution pool is sampled and stored at 2°
15 to 8° C until it is further processed. Optionally, the column can be regenerated and reused.

Example 4 - Purification of Protein with Hydroxyapatite Chromatography

Ceramic-Hydroxyapatite (Ceramic-HA) is a synthetic form of calcium phosphate consisting of spheroidal macroporous particles with high mechanical strength. Ceramic-HA separates proteins with a wide range of charges and isoelectric points largely on the basis of
20 charge interactions. Factor IX is an acidic protein that binds to Ceramic-HA at approximately neutral pH. Typically, acidic proteins are eluted from Ceramic-HA by the addition of phosphate to the buffer solution. The concentration of phosphate required for elution varies, depending upon the properties of the molecule of interest, thereby allowing differential elution of bound proteins. Ceramic-HA is used in the factor IX purification process to remove inactive
25 factor IX, and other contaminants, in the Matrex Cellufine Sulfate elution pool and to exchange the eluate buffer to one compatible with the final chromatographic step. Because the final chromatography step is immobilized metal affinity chromatography, the buffers used for elution of Ceramic-HA are selected to be compatible with IMAC. This avoids an in-process diafiltration or other buffer exchange procedure. Buffers such as Tris, glycine, histidine are not
30 compatible with IMAC because of disruption of the metal ion-immobilized ligand interaction. Elution of the Ceramic-HA column with phosphate buffers avoids such complications.

In preparation for loading, the Ceramic-HA column is equilibrated with 50 mM TRIS, 500 mM NaCl, pH 7.5. The Matrex Cellufine Sulfate elution pool is titrated to pH 7.5 with dilute HCl and loaded directly onto the Ceramic-HA column. Upon completion of loading, the

column is washed with buffer (0.5 M NaCl, 50 mM TRIS, pH 7.5) to ensure that all of the load has passed through the column and that loosely bound contaminants are removed from the column. Next, the column is washed with 50 mM K_2HPO_4 , 185 mM NaCl, pH 7.2 to remove inactive forms of factor IX from the process stream.

- 5 Upon completion of the wash steps, the bound factor IX is eluted with 500 mM K_2HPO_4 , 200 mM NaCl, pH 7.2, and the factor IX eluate is collected as a single UV-absorbing elution pool. The eluate pool is sampled and stored at 2 to 8 °C until it undergoes further processing. Optionally, the column can be regenerated and reused.

Example 5 - Purification of Protein by Immobilized Metal Affinity Chromatography

- 10 Fractogel-EMD-Chelate is composed of a methacrylate polymer derivatized with iminodiacetic functional groups to which transition-state metal ions can be bound. In preparation for use in the purification process, the resin is charged with copper ions using a solution of copper sulfate. Proteins capable of interacting with the immobilized copper ions are retained on the column, and non-interacting contaminants pass through in the unbound
15 fraction. Bound proteins are eluted from the resin using solutions containing imidazole. A Fractogel-EMD-Chelate-Cu(II) step can be used in the protein purification process to remove from the process stream contaminants that do not bind to the immobilized metal ion or that require higher concentrations of imidazole for elution than those required by factor IX. The term IMAC (immobilized metal affinity chromatography) is also used to denote this
20 chromatography step.

- In preparation for loading, the uncharged (no immobilized metal ion) Fractogel-EMD-Chelate column is washed with 100 mM acetic acid, 500 mM NaCl, pH 4.0 and is subsequently charged with 200 mM $CuSO_4$, 500 mM NaCl. Loosely bound copper ions are removed by washing the charged resin with 100 mM acetic acid, 500 mM NaCl, pH 4.0,
25 followed by 200 mM imidazole, 500 mM NaCl, pH 7.1. The Fractogel-EMD-Chelate-Cu(II) resin is then equilibrated in 200 mM K_2HPO_4 , 200 mM NaCl, pH 7.1 (Equilibration V). The Ceramic-HA elution pool is loaded directly onto the equilibrated Fractogel-EMD-Chelate-Cu(II) column.

- Upon completion of loading, the column is washed with equilibration buffer to ensure
30 that all of the load has passed through the column. Factor IX bound to the resin is eluted using 20 mM K_2HPO_4 , 15 mM imidazole, 100 mM NaCl, pH 7.1. The Fractogel-EMD-Chelate-Cu(II) eluate is collected as a single UV-absorbing pool. After collection, the elution pool is diluted with 20 mL of 500 mM EDTA, pH 8.0 per liter of column eluate. The diluted elution pool is stored at room temperature until it undergoes further processing.

**Example 6 - Concentration and Formulation of Protein by
Ultrafiltration/Diafiltration #2**

To transfer the factor IX to a buffer of choice, a combination ultrafiltration/diafiltration step is used. Tangential-flow UF/DF is a non-chromatographic separation method that can be used to concentrate and buffer-exchange substances in solution. A feedstream is directed parallel to the surface of a selectively permeable membrane, and pressure is applied to the retentate side of the membrane outlet to effect transport of water and solutes at the membrane surface on the basis of their relative permeability. Under these circumstances, low-molecular-weight feedstream components pass freely through the membrane pores into the permeate fraction, and higher-molecular-weight substances (*e.g.*, factor IX) are retained by the membrane and constitute the retentate fraction. In this manner, water and buffer salts can be removed from the Fractogel-EMD-Chelate-Cu(II) elution pool, and the factor IX can be concentrated to a target concentration.

Optionally, a spiral-wound cartridge component of the tangential-flow system is first equilibrated with 10 mM histidine, 260 mM glycine, 1% sucrose, pH 6.8. The Fractogel-EMD-Chelate-Cu(II) elution pool (previously diluted with 500 mM EDTA) is then transferred to the stainless-steel retentate pressure vessel of the tangential-flow apparatus in preparation for protein concentration.

After the transfer is completed, the retentate solution is pumped continuously from the pressure vessel through the spiral-wound cartridge and back to the pressure vessel under a net positive transmembrane pressure. The volume of the retentate is monitored continuously during this operation by measuring the permeate fraction volume using a graduated collection vessel.

When the target retentate volume is reached, the retentate pool is diafiltered into the buffer of choice. During this operation, diafiltration buffer is pumped into the pressure vessel at the same rate at which permeate flows from the system, thereby maintaining a constant retentate volume.

After completion of the diafiltration step, the retentate fraction is concentrated to a target volume using ultrafiltration. The outflow fraction from the retentate pressure vessel is stopped, and the retentate fraction in the spiral-wound cartridge is flushed into the retentate pressure vessel with a target volume of the buffer of choice. The concentrated, diafiltered factor IX product is recovered from the pressure vessel by pumping into tared pool bottles.

The factor IX product pool is diluted with 10 mM histidine, 260 mM glycine, 1% sucrose, 1% polysorbate 80, pH 6.8 to a final concentration of 0.005% polysorbate 80. The product is then mixed thoroughly and filtered through a 0.2- μ m filter (previously equilibrated

in 10 mM histidine, 260 mM glycine, 1% sucrose, 0.005% polysorbate 80, pH 6.8 into depyrogenated Teflon bottles. The protein is then sampled, labeled, frozen quickly in liquid nitrogen, and stored at -80° C

While the present method of the invention is exemplified by purification of recombinantly-produced factor IX from transformed host cells, the method is also amenable to purification of factor IX naturally occurring within a cell and can be used to purify proteins from solution or from plasma, cell homogenates, cell culture supernatants, or isolated cellular sub-fractions. While the present invention has been described in terms of specific methods and compositions, it is understood that variations and modifications will occur to those skilled in the art upon consideration of the present invention.

Numerous modifications and variations in the invention as described in the above illustrative examples are expected to occur to those skilled in the art and, consequently, only such limitations as appear in the appended claims should be placed thereon. Accordingly, it is intended in the appended claims to cover all such equivalent variations which come within the scope of the invention as claimed.

WHAT IS CLAIMED:

1. A method for purification of factor IX in a solution comprising the steps of:
applying said solution to an anion exchange resin,
eluting said anion exchange resin with a first eluant to form a first eluate,
applying said eluate to a heparin-like resin,
eluting said heparin-like resin with a second eluant to form a second eluate,
applying said second eluate to an hydroxyapatite resin, and
eluting said hydroxyapatite resin with a third eluant to form a third eluate.
2. The method of claim 1, further comprising the step of:
washing said anion exchange resin with a first wash prior to eluting with said first eluant.
3. The method of claim 2, wherein said first wash comprises a solution selected from the group consisting of: sodium chloride, potassium chloride, sodium sulphate, sodium phosphate, or potassium phosphate.
4. The method of claim 3, wherein said first wash is 200 mM sodium chloride.
5. The method of claim 1, further comprising the steps of:
applying said third eluate to an immobilized metal affinity resin, and
eluting said immobilized metal affinity resin with a fourth eluant to form a fourth eluate
6. The method of claim 1, wherein said first eluant comprises a divalent cation selected from the group consisting of: calcium, magnesium, manganese, strontium, zinc, cobalt, and nickel.
7. The method of claim 6, wherein said first eluant is 10 mM calcium.
8. The method of claim 1, wherein said anion exchange resin has a positively charged group which is a member selected from the group consisting of: diethyleaminoethane (DEAE), polyethyleneimine (PEI), and quaternary aminoethane (QAE).
9. The method of claim 8, wherein said anion exchange resin is Q-Sepharose Fast Flow.
10. The method of claim 1, wherein said heparin-like resin has a negatively charged group which is a member selected from the group consisting of heparin, sulphated esters of cellulose, sulphypropyl (SP), carboxyl, and carboxy methyl.
11. The method of claim 10 wherein said heparin-like resin is Matrex Cellufine Sulfate.
12. The method of claim 1, wherein said second eluant is a member selected from the group consisting sodium chloride and potassium chloride.
13. The method of claim 12, wherein said second eluant is 50mM Tris, 0.50M NaCl, pH 8.0.

14. The method of claim 1, wherein said third eluant is a member selected from the group consisting of phosphate and sulphate.
15. The method of claim 14, wherein said third eluant is 0.5M potassium phosphate, 0.2M NaCl, pH 7.2.
16. The method of claim 1, wherein said hydroxyapatite resin is member selected from the group consisting of ceramic-hydroxyapatite and BioGel-HT.
17. The method of claim 16, wherein said hydroxyapatite resin is ceramic-hydroxyapatite.
18. The method of claim 5, wherein said immobilized metal affinity resin is a member selected from the group consisting of Fractogel-EMD-Chelate, Chelating-Sepharose, Matrex Cellufine Chelate, and POROS 20MC.
19. The method of claim 5, wherein said immobilized metal affinity resin is Fractogel EMD-Chelate.
20. The method of claim 5, wherein said fourth eluant is a displacer.
21. The method of claim 20, wherein said displacer is a member selected from the group consisting of imidazole, EDTA, EGTA, glycine, histidine, and Tris.
22. A method for purification of factor IX in a solution comprising the steps of:
applying said solution to an anion exchange resin,
eluting said anion exchange resin with a first eluant to form a first eluate,
applying said first eluate to an hydroxyapatite resin, and
eluting said hydroxyapatite resin with a third eluant to form a third eluate.
23. The method of claim 22, further comprising the step of:
washing said anion exchange resin with a first wash prior to eluting with said first eluant.
24. The method of claim 22, further comprising the steps of:
applying said third eluate to an immobilized metal affinity resin, and
eluting said immobilized metal affinity resin with a fourth eluant to form a fourth eluate.
25. A method for purification of factor IX in a solution comprising the steps of:
applying said solution to an anion exchange resin,
washing said anion exchange resin with a first wash, and
eluting said anion exchange resin with a first eluant to form a first eluate,
wherein said first eluant has a conductivity lower than the higher of said first wash conductivity and of said solution conductivity.

26. The method of claim 25, wherein said first wash comprises a solution selected from the group consisting of: sodium chloride, potassium chloride, sodium sulphate, sodium phosphate, or potassium phosphate.
27. The method of claim 26, wherein said first wash is 200 mM sodium chloride.
28. A method for purification for factor IX in a solution comprising the steps of:
applying said solution to an hydroxyapatite resin,
washing said hydroxyapatite resin with a first phosphate buffer to remove inactive forms of factor IX,
eluting said hydroxyapatite resin with a second phosphate buffer, wherein said second phosphate buffer has a higher phosphate concentration than said first phosphate buffer.
29. The method of claim 28, wherein said first phosphate buffer is a sulphate buffer.
30. The method of claim 28, wherein said first phosphate buffer is 50 mM potassium phosphate, 0.185 M NaCl, pH 7.2 and said second phosphate buffer is 0.5 M potassium phosphate, 0.2 M NaCl, pH 7.2.
31. A factor IX produced by the method of claim 1.
32. A factor IX produced by the method of claim 5.
33. A factor IX produced by the method of claim 22.
34. A factor IX produced by the method of claim 24.
35. A factor IX produced by the method of claim 25.
36. A factor IX produced by the method of claim 28.
37. A factor IX having a specific activity of 240-400 U/mg.
38. The factor IX of claim 37, wherein said specific activity is about 240 U/mg.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/07305

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N9/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 363 126 (LILLY CO ELI) 11 April 1990	25-27, 35,37,38
Y	see the whole document	1-24, 31-34

X	THROMBOSIS RESEARCH, vol. 49, no. 2, 1988, pages 277-286, XP002013371	37,38
	A. BENNY ET AL: "Comparative separation of human plasma proteins on polysulphated chromatographic media"	
Y	see the whole document	1-21,31, 32

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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

 Int ional Application No
 PCT/US 96/07305

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CHROMATOGRAPHY, vol. 632, 1993, AMSTERDAM NL, pages 1-10, XP002013372 D. JOSIC ET AL: "Isolation of plasma proteins from the clotting cascade by heparin affinity chromatography"	25-27, 35,37,38
Y	see the whole document	1-24, 31-34

X	HAEMOSTASIS, vol. 1, 1972, pages 2-22, XP000602120 P. REEKEERS AND H. HEMKER: "Purification of blood coagulation factors II, VII, IX, X from bovine citrated plasma"	25-30, 35-38
Y	cited in the application see the whole document	1-21,31, 32

X	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 71, 1976, pages 145-154, XP000602032 H. SUOMELA: "Human coagulation factor IX"	1-4, 6-17, 25-31, 35-38
Y	see the whole document	5,18-21, 32

X	BIOTECHNOLOGY OF BLOOD PROTEINS, vol. 227, 1993, pages 63-68, XP000602055 P. FELDMAN ET AL: "Preparation of a high purity factor IX concentrate using metal chelate affinity chromatography"	25-27, 35,37,38
Y	cited in the application see the whole document	5,18-21, 24,32,34

X	EP,A,0 617 049 (SCLAVO SPA ;AIMA DERIVATI SPA (IT)) 28 September 1994	25-27, 35,37,38
Y	see the whole document	5,18-21, 24,32,34

X	WO,A,89 05650 (CENTRAL BLOOD LAB AUTHORITY) 29 June 1989	37,38
Y	see the whole document	5,18-21, 24,32,34

P,X	BLOOD, vol. 86, no. 10, November 1995, page 870a XP002013377 W. FOSTER ET AL: "Development of a process for purification of recombinant human factor IX"	1-21, 25-32, 35-38
	see abstract 3468 -----	

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/07305

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0363126	11-04-90	US-A-	4981952	01-01-91
		AT-T-	106406	15-06-94
		AU-B-	635222	18-03-93
		AU-A-	4251989	12-04-90
		CA-A-	1314011	02-03-93
		DE-D-	68915675	07-07-94
		DE-T-	68915675	20-10-94
		ES-T-	2054019	01-08-94
		IE-B-	63765	14-06-95
JP-A-	2200180	08-08-90		

EP-A-0617049	28-09-94	US-A-	5378365	03-01-95

WO-A-8905650	29-06-89	AU-A-	3032989	19-07-89
		DE-A-	3881895	22-07-93
		DE-T-	3881895	02-12-93
		EP-A-	0391974	17-10-90
		JP-B-	6000064	05-01-94
		JP-T-	3501085	14-03-91
		NO-B-	178882	18-03-96
US-A-	5445958	29-08-95		

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